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EFFECTS OF VARIOUS ELICITORS ON THE ACCUMULATION AND SECRETION OF ISOFLAVONOIDS IN WHITE LUPIN

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Key Word Index—*Lupinus albus*; Leguminosae; isoflavonoids; elicitation; genistein; 2′-hydroxygenistein; glucosides; prenylated derivatives.

Abstract—Roots of white lupin seedlings were exposed to yeast extract, chitosan, *Rhizobium loti* suspension and $CuCl_2$; and the isoflavonoids of their tissue extracts as well as their exudates were analysed by HPLC. Apart from specific responses characteristic of different elicitors, most treatments produced dramatic increases in the amounts of genistein and 2′-hydroxygenistein monoprenyls in root tissues, as well as major increases of all isoflavonoids in the exudates. The effect of symbiotic stress, in comparison to other stresses, is discussed in relation to the specificity of isoflavone production in white lupin. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Phytoalexins and phytoanticipins [1, 2], are considered to be defence chemicals that are produced by plant tissues in response to various stress factors [1, 3]. These metabolites are considered to be part of the plant defence system resulting from rapid, but transient transcriptional activation of key enzymes involved in their biosynthesis [4, 5]. Recent work seems to indicate that elicitor-induced secondary metabolite synthesis is similar to that observed during microbial infection [6, 7]. In most cases, however, the resulting response of elicitation is the *de novo* synthesis of a variety of phytoalexins with diverse chemical structures, including flavonoids, terpenoids and alkaloids, to mention a few [1, 8, 9].

In contrast with most legumes, which accumulate post-infectional phytoalexins in response to microbial attack, the roots of white lupin (*Lupinus albus* L.) exhibit the constitutive expression of a complex pattern of prenylated isoflavones (Table 1) that are derived from genistein and 2′-hydroxygenistein [10]. The latter compounds may thus be considered pre-infectional metabolites, some of which have been reported to be fungitoxic [11, 12]. Most isoflavonoids and their prenylated derivatives represent the predominant phytoalexins of the Fabaceae [9, 13], and their antimicrobial activity seems to increase with increasing level of prenylation [8].

Apart from one report in the literature, on the effect of $CuCl_2$ on the accumulation of isoflavone aglycones and their monoprenylated derivatives in white lupin seeds [14], there is an evident lack of information as to the effect of elicitors on the biosynthesis and accumulation of lupin seed isoflavonoids during germination and development. This prompted us to investigate the effect of the commonly used elicitors, $CuCl_2$, chitosan and yeast extract, on the isoflavonoid pattern of this system. In addition, the fact that daidzein is known to act as a signal molecule in *Rhizobium*–legume symbioses [15, 16], and that some phytoalexins are known to be synthesized upon symbiotic elicitation [17, 18], led us to include a compatible lupin symbiont, *R. loti* among the elicitors used. This may allow us to distinguish between the 'symbiotic' phytoalexins and the 'classical' phytoalexins.

RESULTS AND DISCUSSION

As with most other legumes [19], white lupin has the ability to establish a symbiotic association with *R. loti*. Two-day-old germinating lupin seeds were treated with *R. loti*, yeast extract, and chitosan as biotic elicitors, and $CuCl_2$ as an abiotic elicitor. Tissue extracts were analysed for the accumulation and secretion of isoflavonoids at 2, 5 and 8 days after treatment. The HPLC protocol utilized in this study allowed for the separation and the unambiguous characterization of the 11 individual isoflavonoids analyzed in a single chromatographic run. However, in order to facilitate data presentation and discussion, the individual compounds derived from either

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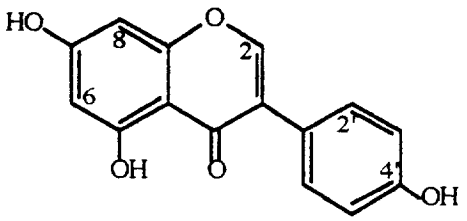
Table 1. *Lupinus albus* isoflavonoid groups, their trivial and generic names



Isoflavone numbering system

Trivial name	Generic name
Glucosides	
Genistein glucoside	5,7,4′-Trihydroxyisoflavone 7-*O*-glucoside
2′-Hydroxygenistein glucoside	5,7,2′,4′-Tetrahydroxyisoflavone 7-*O*-glucoside
Aglycones	
Genistein	5,7,4′-Trihydroxyisoflavone
2′-Hydroxygenistein	5,7,2′,4′-Tetrahydroxyisoflavone
Genistein monoprenyls*	
Wighteone	6-Prenyl-5,7,4′-trihydroxyisoflavone
Isowighteone	3′-Prenyl-5,7,4′-trihydroxyisoflavone
Lupiwighteone	8-Prenyl-5,7,4′-trihydroxyisoflavone
2′-Hydroxygenistein monoprenyls	
Luteone	6-Prenyl-5,7,2′,4′-tetrahydroxyisoflavone
Licoisoflavone A	3′-Prenyl-5,7,2′,4′-tetrahydroxyisoflavone
Diprenyls	
Lupalbigenin	6,3′-Diprenyl-5,7,4′-trihydroxyisoflavone
2′-Hydroxylupalbigenin	6,3′-Diprenyl-5,7,2′,4′-tetrahydroxyisoflavone

*Prenyl = 3,3-dimethylallyl.

genistein or 2′-hydroxygenistein (Table 1) were grouped into glucosides, aglycones, and mono- and diprenyl derivatives.

Isoflavonoids of stressed roots and their exudates. There was a slight, but significant, increase in the total isoflavonoids observed in seedling root tissues after treatment for 5 days with various elicitors, except for the *R. loti* and 300 μM $CuCl_2$ treatments which were similar to their controls (Table 2). The amounts of glucosides (*ca* 88–99%) in root tissues appear to constitute the bulk of isoflavonoids detected in both control and elicitor treatments (Table 2), as was previously reported for sterile lupin seedling roots [20]. However, there were dramatic increases in the amounts of genistein and 2′-hydroxygenistein monoprenyls in all stress treatments, which ranged from 6- to 13-fold and 2- to 98-fold above the controls, respectively. In addition, the increase in total isoflavonoids identified in root exudates (Table 3) appear to have taken place at the expense of the glucosides and, to a lesser extent, of the aglycones in root tissues (Table 2). This is best illustrated with *R. loti*-stressed roots, where the decrease in all isoflavonoids between seedling roots aged day 7 (Table 2) and day 10 (*ca* 1.69 nmol mg^{-1} fr. wt) was mostly at the expense of the glucosides (data not shown), which correlates with the dramatic increase in the total amount of prenylated derivatives found in the exudates (Tables 3, 4). A continuous increase is observed in the accumulation and the secretion of total prenylated isoflavones at 2, 5 and 8 days after treatment with all elicitors (Table 4). Similar results have been reported for chickpea cell suspension cultures [21] where elicitation by yeast extract resulted in an increased ratio of phytoalexin aglycones to their glucoconjugates. Furthermore, the predominant accumulation of monoprenylated compounds, especially those of 2′-hydroxygenistein, in stressed roots (Tables 2 and 4) suggests a role for these metabolites in white lupin response to such elicitors as $CuCl_2$, yeast or chitosan. In fact, the prenylated isoflavonoids luteone and licoisoflavone A (both 2′-hydroxygenistein monoprenyls) have been shown to be fungitoxic [11, 12]. Furthermore, these 2′-hydroxygenistein monoprenyls have been reported to accumulate in the roots of white lupin upon $CuCl_2$ elicitation [14].

On the other hand, the total amounts of isoflavonoids secreted into the medium were higher for all elicitor treatments as compared with the controls (Table 3). However, considering the high concentration of yeast extract used as an elicitor, it is reasonable to assume that the release of glucosides and aglycones in the exudates (Table 3) may have been the result of damaged cell membranes, thus leading to passive leakage of these derivatives [22], as evidenced by the browning of root tissues at day 7. It has previously been reported that a 0.25% yeast extract and 2 mM $CuCl_2$ exerted very potent elicitor activities

Table 2. Isoflavonoid content of stressed lupin seedling-roots*

Isoflavonoid groups†	Control medium	*Rhizobium loti*	Yeast extract	Chitosan		$CuCl_2$	
				Control	0.005%	Control	300μM
Glucosides	3.88	3.31	3.2	2.15	3.64	4.98	4.73
Aglycones	0.43	0.41	1.06	0.58	0.44	0.46	0.80
Gen. MonoPr.	0.03	0.19	0.29	0.02	0.20	0.02	0.27
2′-OH Gen. MonoPr.	0.01	0.02	0.10	0.03	0.25	0.01	0.98
Diprenyls	0.00	0.06	0.03	0.01	0.02	0.01	0.03
Total identified‡	4.35[b]	3.99[b]	4.68[a]	2.79[e]	4.55[f]	5.48[b]	6.81[b]
Gen. derivatives§ (%)	85.7	82.9	72.1	83.1	77.2	75.4	55.6

*Two-day old seedlings were treated for 5 days in the following control and stress media: control medium [28]; yeast extract, 1.0% (w/v) yeast extract in control medium; *R. loti*, *R. loti* suspension A_{600} = 0.024 in control medium; chitosan control, 0.01% aq. HOAc soln (v/v) buffered with NaOAc to pH 6.0; chitosan, 0.005% (w/v) in chitosan control soln; $CuCl_2$, 0.3 mM in H_2O; $CuCl_2$ control, H_2O acidified with HCl to pH 4.3. Values are averages of duplicate samples, each of three dissected roots, expressed in nmol mg^{-1} fr. wt.

†See Table 1 for individual isoflavones included in each group. Gen., genistein; MonoPr., monoprenyls.

‡[a–f], standard deviations, with levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

§%Genistein derivatives in total isoflavonoids; the remaining constitutes the 2′-hydroxygenistein derivatives.

Table 3. Isoflavonoid content of root-exudates from stressed lupin seedlings*

Isoflavonoid groups†	Control medium	*Rhizobium loti*	Yeast extract	Chitosan		$CuCl_2$	
				Control	0.005%	Control	300 μM
Glucosides	0.64	0.38	2.24	2.31	5.35	0.59	5.61
Aglycones	0.48	1.84	4.1	13.17	25.29	1.11	23.63
Gen. MonoPr.	0.76	7.30	6.91	0.12	0.64	1.36	3.83
2′-OH Gen. MonoPr.	0.15	0.36	7.17	0.16	2.69	0.84	10.38
Diprenyls	0.11	0.28	0.28	0.07	0.20	0.15	0.03
Total identified‡	2.14[b]	10.16[c]	20.7[f]	15.83[c]	34.17[c]	4.05[f]	43.48[e]
Gen. derivatives§ (%)	77.5	80.2	40.7	97.2	51.8	61.0	32.7

*Two-day old seedlings were treated for 5 days in the following control and stress media: control medium [28]; yeast extract, 1.0% (w/v) yeast extract in control medium; *R. loti*, *R. loti* suspension A_{600} = 0.024 in control medium; chitosan control, 0.01% aq. HOAc soln (v/v) buffered with NaOAc to pH 6.0; chitosan, 0.005% (w/v) in chitosan control soln; $CuCl_2$, 0.3 mM in H_2O; $CuCl_2$ control, H_2O acidified with HCl to pH 4.3. Values are averages of duplicate samples, each of the root exudates of nine seedlings; expressed in nmol $seedling^{-1}$.

†See Table 1 for individual isoflavones included in each group. Gen., genistein; MonoPr., monoprenyls.

‡[a–f], standard deviations, with levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

§%Genistein derivatives in total isoflavonoids; the remaining constitutes the 2′-hydroxygenistein derivatives.

in chickpea cell cultures [23] and lupin seeds [14], respectively. On the other hand, the increase in secretion of both glucosides and aglycones of chitosan treatments does not appear to be of the same magnitude as in yeast treated roots (Tables 2 and 3). This may be attributed to the sensitivity of lupin roots to as low as 0.01% HOAc used as the chitosan control, which resulted in increases of 3.6- and 27-fold in the glucosides and aglycones, respectively, above those of the control medium (Table 3). However, a comparison of the effect of different stresses on the isoflavonoid pattern of the root exudates reveals some characteristic responses. Among these are the 10-fold increase in the genistein monoprenyls of *R. loti*-stressed roots; a 3.5- to 48-fold increase in the glucosides, aglycones, genistein- and 2′-hydroxygenistein monoprenyls in yeast-treated tissues; and a 3- to 20-fold increase in the latter constituents in the $CuCl_2$-stressed root exudates (Table 3). These data indicate that, except for *R. loti*, other stresses result in a significant decrease in the relative abundance of genistein derivatives (Tables 2 and 3). On the other hand, symbiotic-elicitation exhibits an increased secretion of genistein monoprenyls, which is in contrast with the increased accumulation and secretion of 2′-hydroxygenistein monoprenyls after elicitation with $CuCl_2$, yeast extract or chitosan.

Does Rhizobium loti elicit a specific response in white lupin? The results obtained with *R. loti* stressed roots (Table 2) clearly indicate that early infection of white lupin roots with this symbiont resulted in a relatively low accumulation of isoflavonoids in root tissues as compared with their level of secretion into the medium. These results are comparable to soybean

Table 4. Accumulation and secretion of total prenylated isoflavonoids by lupin roots stressed with biotic and abiotic elicitors*

Treatments†	Seedling roots			Root exudates		
	D2	D5	D8	D2	D5	D8
Control medium‡	0.02^{d}	0.04^{c}	0.10^{d}	0.44^{f}	1.02^{d}	1.70^{f}
R. loti suspension	0.08^{c}	0.27^{a}	0.27^{b}	0.91^{f}	7.94^{e}	8.91^{a}
Yeast extract (1% w/v)	0.30^{e}	0.42^{b}	1.02^{b}	3.74^{f}	14.36^{f}	53.63^{c}
Chitosan (0.005% w/v)	0.50^{c}	0.47^{b}	ND§	0.45^{f}	3.53^{f}	ND
300 μM $CuCl_2$	0.66^{b}	1.28^{d}	2.53^{c}	2.97^{a}	14.24^{e}	22.52^{f}

*Two-day-old seedlings were treated for 2, 5 and 8 days, and analysed for prenylated isoflavonoids in the roots and their exudates; D, days after treatment. Values are averages of duplicate samples, each of three dissected roots, or of exudates of nine roots; expressed in nmol mg^{-1} fr. wt and nmol $seedling^{-1}$ for root tissues and exudates, respectively. Total prenylated isoflavonoids includes all mono- and diprenylated isoflavones shown in Table 1. a–f, standard deviations with levels of: a, $<5\%$; b, $<10\%$; c, $<15\%$; d, $<20\%$; e, $<25\%$; f, $>25\%$.

†Control medium [28]; *R. loti*, *R. loti* suspension $A_{600} = 0.024$ in control medium; yeast extract, 1.0% (w/v) yeast extract in control medium; chitosan, 0.005% (w/v) in 0.01% aq. HOAc soln (v/v) buffered with NaOAc to pH 6.0; $CuCl_2$, 0.3 mM $CuCl_2$ soln.

‡The chitosan and $CuCl_2$ controls were similar to those of the control medium.

§Not determined due to death of tissues after treatment for 8 days.

where the phytoalexin, glyceollin I secretion was 50-fold higher after inoculation with *B. japonicum*, as compared with that of control seedlings [17]. Similar results were reported for *Medicago sativa* [18] and *Vicia sativa* [24] after elicitation with their compatible rhizobia. However, the fact that the rate of secretion of glyceollin I was reported to be several times lower after symbiotic infection than after pathogenic infection [25] further corroborates our results. However, recent investigation of the effect of wighteone (a genistein monoprenyl) on the growth rate of *R. loti* indicated a significant inhibition of the *in vitro* growth of this symbiont (H. Gagnon, unpublished results), which contrasts with the observation that luteolin, the *nod-gene* inducer of *R. meliloti*, stimulated the *in vitro* growth of the symbiont [26]. Therefore, our results seem to suggest that genistein monoprenyls may in fact act as "symbiotic phytoalexins" that could be involved in the control of early *Rhizobium*-infection of lupin roots. This situation compares with the phytoalexin, glyceollin I [17,25] which was also reported to inhibit the *in vitro* growth of the soybean symbiont, *B. japonicum* [27]. Work is currently in progress to characterize which of the lupin isoflavonoids is responsible for the induction of nodulation genes in *Rhizobium loti*.

EXPERIMENTAL

Plant material. Seeds of white lupin, *Lupinus albus* L. cv. Kievskij (Fabaceae) were surface sterilized by flaming in EtOH for 10 s, then shaking with a water–bleach solution (4:1) containing 0.05% Tween 20 on a rotary shaker at 250 rpm for 30 min, followed by thorough rinsing with sterile water. Surface sterilized seeds were allowed to imbibe water for 24 hr while shaking (175 rpm) under aseptic conditions. Following imbibition ($t = 24$ hr) the seeds were placed with the emerging root facing down on vermiculite in polycarbonate containers under aseptic conditions, and incubated under the following conditions: 16 hr-light (*ca* 400 E m^{-2} s^{-1}) and 8 hr-dark, at 26°/20°C, and 50% relative humidity. At $t = 48$ hr, the seedlings with straight emerging roots (10 to 25 mm) were mounted aseptically on stainless steel grids over glass Petri dishes containing nutrient media (with or without elicitor). Duplicate samples, each consisting of three root systems, as well as the exudate-containing culture media of nine seedlings were harvested for analysis at 2, 5 and 8 days after treatment.

Nutrient and stress media. All media were prepared with deionized water and were filter-sterilized prior to use. The control medium consisted of a nutrient solution [28] in which the N-containing salts were replaced with N-free salts, adjusted to pH 6.5. The *R. loti* medium was prepared from a single colony (ATCC # 35173) grown in liquid YEM medium [29] on a rotary shaker (300 rpm) at 28° to an A_{600} of 1.2 units, then diluted (1/50, v/v) with control medium to 0.024 units. The yeast extract was dissolved (1%, w/v) in control medium. Chitosan (β-1,4-linked glucosamine polymer, Sigma) was solubilized (0.5%, w/v) in 1% aq. HOAc, adjusted to pH 6.0 with NaOAc and diluted to 0.005% (w/v); 1% buffered HOAc, pH 6.0 was diluted similarly and used as a control. $CuCl_2$ was prepared as a 0.3 M aq. soln (pH 4.3) and diluted as required; sterile water with the same pH served as a control. Additional controls for the chitosan and $CuCl_2$ treatments were used in order to account for the buffering capacity of the incubation medium, or the altered pH upon solubilization of both compounds, respectively.

Preparation of tissue extracts and root exudates. Whole root systems were homogenized with washed sand (5:1, w/w) and 80% MeOH (1:4, w/v) in Eppendorf tubes at room temp., then centrifuged for 2 min

(10,000 rpm). The pellets were further extracted 2× with 80% and 1× with 100% MeOH. The combined methanolic extracts were evapd in a Speed Vac concentrator, and the residue was redissolved in an aliquot of 80% MeOH equivalent to the original tissue wt, then centrifuged prior to HPLC analysis. Root exudates were adjusted to pH 4.5, before being extracted 2× with an eq. vol. of EtOAc, and the combined organic layers were evapd at 40° under vacuum. The residue was redissolved first with 80%, followed by 100% MeOH, and the combined extracts were lyophilized and redissolved in a measured vol. of 80% MeOH.

HPLC analysis of isoflavonoids. Lupin isoflavonoids (Table 1) were chromatographed on a Merck™ RP-C_{18} LiChrospher 100 column (250 × 4 mm i.d., 5 μm particle size) as previously described [30]. The elution protocol was carried out at a flow rate of 1 ml min^{-1} using 45% solvent A (0.5% methanolic HOAc) in 55% solvent B (0.5% aq. HOAc) for 2 min, followed by a gradient increase to 100% solvent A in 23 min, after which isocratic conditions were maintained for a further 10 min. Both qualitative and quantitative analyses, as well as the calibration curves of different isoflavonoids, were achieved using the software Baseline™ 810 and Excel™.

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